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Phaseolotoxin Biosynthesis: Molecular Mechanisms of Thermoregulation

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)
The production phaseolotoxin (PT) and PT-resistant ornithine carbamoyl-transferase (encoded by *argK* of *Pseudomonas syringae* pv. *phaseolicola* are thermoregulated; both are produced at 18°C, but neither is produced at 30°C. At 30°C (but not at 18°C) the bacterium produces a DNA-binding protein that binds (*in vitro*) to sites in the upstream region of *argK*. Our hypothesis is that this protein is a transcription factor (TP) which negetively regulates *argK* and PT genes. The main objectives of this grant were to obtain complete sequences of the genes harbored by the PT gene cluster and to characterize them; and secondly, to purify the TP and clone and sequence the gene that encodes it. We have sequenced and characterized PT gene loci, *phtH*, *phtG*, *phtF*, *phtD*, *phtC* and most of *phtB* (total sequence, 16, 163-bp). Most of the genes in these loci show no homology to any genes in the databases. *phtF* the only gene in *phtF* locus harbors a DNA-binding motif. We have purified TP, and obtained its N' terminal amino acid sequence on which was based the oligonucleotide probe used in screening of the genomic library of *P.s.pvphaseolicola*. The screening yielded a clone which harbored a gene encoding the TP. It's sequence showed 54% identity to a DNA-binding protein from *E.coli*.

14. SUBJECT TERMS
Thermoregulation of phaseolotoxin production, DNA sequences of Pheseolotoxin biosynthetic genes, repressor protein binding of DNA motifs in the *argK* gene of *Pseudomonas syringae* pv. *Phaseolicola,* cloning of the repressor gene, gel shift assays, PCR, footprinting.

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CONTRACT/GRANT NUMBER: DAAH04-96-1-0032

REPORT TITLE: Final Progress Report for ARO Grant.
"Phaseolotoxin Biosynthesis: Molecular Mechanisms of Thermoregulation".

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SUBMITTED FOR PUBLICATION TO (applicable only if report is manuscript):

Sincerely,

Suresh S. Patil

Enclosure 3

REPORT DOCUMENTATION PAGE (SF298)
(Continuation Sheet)

Final Progress Report

ARO Grant Number: DAAH04-96-1-0032
Title: Phaseolotoxin Biosynthesis: Molecular Mechanisms of Termoregulation
Principal Investigator: Suresh S. Patil
Pacific Biomedical Research Center
University of Hawaii
Honolulu, HI 96822
Period Covered: 05/01/96 through 07/31/2000

Problem Studied:

Pseudomonas syringae pv. *phaseolicola* produces a phytotoxin called phseolotoxin. The pathogen protects itself from phaseolotoxin by producing a toxin-resistant form of its target, ornithine carbamoyltransferase (ArgK). *argK* and genes involved in phaseolotoxin production are coordinately regualted by temperature; at 18°C, phaseolotoxin as well as ArgK are produced, but at 30°C, neither is produced. Previous work has shown that at 28°C (but not at 18°C) , the pathogen produces a protein in culture that specifically binds to DNA motifs in the leader region of *argK* (Rowley et al. 1993). Gel shift and footprinting assays have identified specific nucleotides involved in this binding. Our hypothesis is that the DNA binding protien produced by *P.s.* pv. *Phaseolicola* represses the transcription of *argK* and the phaseolotoxin biosynthetic genes at 30°C. To test this hypothisis we needed to purify the repressor protein, characterize it, and clone the gene that encodes it. We also needed to determine the nucleotide sequence of the entire phaseolotoxin biosynthetic gene cluster. We had previously sequenced locus *phtE*. During the period of this grant we have sequenced additional loci, form the phaseolotoxin gene cluster.

The original specific objectives of the grant were:

1. Clone and characterize the gene that encodes the putative repressor from *P.s.* pv. *phaseolicola*.
2. Determine if the protein encoded by this gene is involved in a regulatory cascade that senses temperature.
3. Determine which of the remaining 7 loci in the pHK120 toxin gene cluster are thermoregulated and examine whether they harbor regulatory DNA motifs. Also, complete the cloning and characterization of the putative peptide synthetase gene and determine if it is thermoregualted.

Summary of the most important results:

DNA Sequencing (Objective 3)

1. Sequencing

Sequencing (Cycle Sequencing, ABI 377) of Loci *phtH, phtG, phtF, phtD,* and *phtC* (Appendix Fig. 1) has been completed by primer walking. So far a total of 16,163 bp have been sequenced, which includes the previously reported (Zhang et al., 1997) sequence of locus *phtE.*

1a) The restriction sites downstream of locus *phtE* (H5, St, B3, H4 and H3) and upstream of *phtE* (St, H6, H9 & B7, P9 and H8) in Fig. 1B are in agreement witht he physical map in Fig. 1A.

1b) We determined the upstream sequence of *phtH* and confirmed that the 641 bp sequence of *argK*, including its promoter region precedes *phtH* on the complementary strand. This sequence matches the *argK* sequenc in the Genbank data base. The promoter region of the *argK* gene overlaps the promoter region of a gene in locus *phtH.*

1c) We have obtained the sequence of the polylinker of pLAFR3 right arm, which precedes the leading region of *argK* and confirmed that the DNA sequence is a portion of pHK120 insert in pLAFR3. We have synthesized primers for the left arm, which will be used to sequence the *phtA* locus in the future.

2. Gene Coding regions in *phtH, phtG, phtF* and *phtC*.

The potential -35 and -10 promoter regions and the predicted prokaryotic rho-independent termination like sequences and other characteristics of potential gene coding regions were determined. The following is a brief summary of the Blast searches of Swiss Prot databases with our sequence:

phtH has three gene coding regions, phtH0, phtH1 and phtH2.
- *phtH*0 from nt 568-912
- *phtH*1 from nt 971-2323
- *phtH*2 from nt 2392-2640

phtG harbors one gene *phtG* (from nt 2769-3611)

phtF harbors one gene *phtF* (from nt 3617-4663)

phtC harbors one gene *phtC* (starts nt 14,644)

phtD harbors three genes (may extend further toward *phtC* (Fig. 1A)
- *dcd-like-D1* (start nt 10,502)
- *dcd-like-D2* (start nt 10,927)
- *PEP-like* (Phosphoenol pyruvate)-*D3* (start nt 11,568)

phtB: sequenced 660 nt (continuing). No homology found using the 660 bp *phtB* sequence.

Aside from some homology *to dcd-like* and *PEP-like* genes in the *phtD* locus, our searches did not reveal significant homology for the genes in *phtC, phtF, phtG* and *phtH* to any sequences in the databases.

3) Determination of DNA-binding sites. We examined the promoter regions of the genes described in section 2 above and found only one conserved motif CTTTG (10 nt) CTTTC (nt 3476-3495), which overlaps the -35 promoter region of the gene *phtF*. Another possible DNA-binding motif is at nt 50 upstream of -35 promoter region of *phtH2* gene. The conserved binding motifs are not the only sites to which the thermoregulatory protein binds as we discovered using the upstream region *of argK*; there are four sites in this region, only one of which has a half motif (CTTTG). Therefore, it is necessary to examine the promoter and upstream sequences of the other genes described above. The best way to do this would be to use PCR fragments as probes from each gene (primers can be easily synthesized since we have complete sequence data on all the genes described above) to conduct gel shift assays.

Purification of the Putative Repressor.

About 0.5 g of protein from the sonicated extract of G-50-1 cells was used for sequential purification using ammonium sulfate precipitation, and Q-Seplarose and Heparin-Agarose chromatography. Briefly, aliquots of dialyzed crude extracts were loaded onto the Q-Sepharose column (2.0X20.0; bed volume 50 ml; equilibrated with buffer A (25 mM TRIS-Cl, pH 8.0; 1mM EDTA; 1.0 mM DTT; 10% glycerol; 0.6 mM PMSF). The proteins were eluted step-wise with buffer A containing increasing amounts of Sodium Chloride (0.1 M to 1.0 M with 0.1 M intervals). Activity of the fractions was determined by performing gel shift assays using the 490-bp fragment from the Thermoregulatory Region (TRR) described previously (Rowley et al., 1993, Rowley et al., 2000). Most of the activity was present in the 0.3 M and 0.4 M NaCl fractions. These fractions were pooled, concentrated, dialyzed, and then loaded onto a Heparin-Agarose column (2.5 x 15 cm, bed volume 40 ml) equilibrated with buffer A containing 50 mM KCl. The column was eluted with buffer A containing increasing amounts of KCl (0.1M to 1.0 M at 0.1 M intervals). Most binding activity was eluted at 0.3 M KCl. These fractions were again pooled , dialyzed with TM buffer (25 mM Tris-Cl pH 8.0, 0.1 mM EDTA, 12.5 mM MgCl2, 1mM DTT, 20% glycerol, 0.1% HP-40, 0.1 M KCl). Fig. 2 (see Appendix for figures) shows the flow chart. Fig. 3 shows the SDS-PAGE analysis of the 0.3 M KCl fraction eluted from the Heparin-Agarose column, and the southwestern blots using the 490-bp labeled (32 P) fragment from the TRR and the 260-bp subfragment of the 490-bp fragment. Both probes hybridize to a 37 kD and a 44 kD band; in addition the 490-bp probe (but not the 260-bp probe) hybridizes to a 170 kD band. The 37 kD and 44 kD protein bands were carefully blotted using polyvinylidene fluoride (PVDF) membrane, the bands were carefully excised and the N' terminal sequence of the protein in each band was determined using an ABI Procise peptide sequencer. The N' terminal sequences of the two proteins are:

37 kD: MDFKDYYKIL

44 kD: MQISVNEFLTP

Screening of the *P.s.* pv. *phaseolicola* genomic library.

The genomic library (Kamdar et al., 1990) of *P.s.* pv. *phaseolicola.*was screened using a redundant synthetic oligonucleotide probe based on the first eight amino acids

from the 37 kD protein. This probe was used to screen the library using standard techniques. The synthetic oligo used as a probe was: 5' ATG GA(C/T) TT(C/T) AA (A/G) GA (C/T) TA (C/T) TA (C/T) AAG 3'.

The first screen showed hybridization to 23 colonies; the second screen showed hybridization to 4 colonies; but the final screen showed hybridization to only a single colony which was reconfirmed. The plasmid in the library clone (in HB 101) was isolated using the QIAGEN Prep Spin column and the plasmid (pLAFR3) was transformed into *E. coli,* DH5α. From the culture of this transformant a larger aliquot of the plasmid DNA (pLAFR3 containing the ~23-Kb insert) was isolated. Fig. 4A, lane 1 shows the gel electrophoresis of DNA in this clone digested with *Pst*I. A gel similar to this was blotted and probed with the synthetic probe used in the library screening. The probe hybridized to a 3 Kb fragment. This fragment was excised from similar lanes from the gel, and Fig. 4A, lane 2 shows the 3 Kb fragment. A blot of the gel in Fig. 4A was subjected to the labeled probe which hybridized to the 3 Kb fragment. (Fig 4B, lanes 1 & 2). Lane 3 in both A & B is the original pLAFR3 plasmid containing the ~23 Kb insert.

The 3 Kb fragment was ligated separately into different plasmids, pT7/pT3α-19 and pUC19 and mobilized into DH5α. Fig 5A shows restriction analysis of plasmid clones isolated from 3 independent DH5α transformants, one pT7/T3 α-19 clone (pRX1) and two pUC19 clones, pRX2 and pRX3, digested with *Sca*I-*Hind*III. Fig 5A, lanes 1, 2, and 3 show a 4.4-Kb band each, which contains the 3-Kb insert plus part of the vector. Fig. 5B, lanes 1, 2, and 3 shows the Southern blot of this gel using the same probe as above. It hybridized to the ~4.4-Kb bands in all 3 lanes.

Sequencing of the pRX3 clone

We used the M13/pUC universal primers to cycle sequence (ABI 377 automatic sequencer) pRX3. Nucleotide sequence alignments showed that the probe sequence matched perfectly with the N' terminal sequence of one of the two genes found in the 3-Kb fragment, (Fig. 6 *cbp-like*). The amino acid sequence alignments of the translated 3-Kb sequence showed that the translated CbpA-like protein sequence had 54% identity with *E. coli* CbpA, 51% identity with *Salmonella typhimurium* CbpA, 34% identity with *Pseudomonas aeruginosa* DnaJ, 34% identity with *E.coli* DnaJ, 41 % identity with *Thermus thermophilus* DnaJ, and 42% identity with *Xyllela fastidiosa* DnaJ.

The sequence of the 3 Kb fragment also showed the presence of another gene, *dnak-like* gene. This gene is transcribed divergently from the *cbpA*-like gene. Amino acid alignment of the DnaK-like protein sequence showed 77% identity to *Pseudomonas aeruginosa* heat-shock protein, 30% identity to *Vibrio cholerae* heat-shock protein 70 family protein, 28% identity with the YegD protein of *E. coli* and 34% identity with DnaK of *Rhodobacter capsulatus*.

Isolation of the 170 kD protein using DNA affinity column.

Our SDS-PAGE analysis of the 0.3 M KCl fraction showed 3 protein bands, 37 kD, 44 kD and 170 kD that hybridized to the 490-bp probe (Fig. 3). The 260-bp probe

hybridized to only the 37 kD and 44 kD bands with 37 kD protein being the most abundant. Because of this we concentrated on the 37 kD protein in our studies. However, we also wanted to purify the 170 kD protein and reasoned that a DNA affinity column using a multimer of the motif GAAAG (5nt) CAAAG found in the 490-bp fragment from the TRR might give us higher quantities of the 170 KD protein. Therefore, we prepared a DNA affinity column. Using established methods (Kadonaga and Tjian, 1986) a 1 ml DNA affinity column was prepared by coupling mutimerized oligonucleotides to CNBr-activated Sepharose 4B (Sigma, St. Louis, MO). The following oligonucleotides and its complementary strand were made.

GTA CAA *GAA AGG* GAT C*CA AAG* TTA A

Complementary oligodeoxynucleotides were annealed, 5' phosphorylated, and ligated to give oligomers of the basic oligodeoxynucleotide unit that ranged from 3-mers to 50-mers. The ligated DNA was then covalently coupled to CNBr-activated Sepharose 4B. The efficiency of coupling of the DNA to the Sepharose was 80%. The concentration of covalently bound DNA in the affinity resin was 34 μg of DNA per ml of resin. 0.2 mg of dialyzed 0.3 M KCl extract eluted from the Heprin-Agarose cloumn was put on the DNA affinity column and eluted with 1 ml of TM buffer containing increasing amounts of KCl (0.2 M to 1.0 M). Most of the binding activity was eluted between 0.3 M-0.7 M KCl.. Binding activity was determined by doing gel shift assays using the 490-bp fragment from TRR as the probe, as done in the experiments reported in Fig. 3. The Active extracts were pooled, dialyzed and analyzed by SDS-PAGE gel electrophoresis. The Southwestern blots of these gels showed only the 170 kD band. Thus the DNA affinity chromatography using the motif mentioned above appears to bind the 170 kD protein with high affinity but has less affinity for the 37 kD and 44 kD proteins.

In summary we have accomplished the major objectives of the ARO grant:

a) We have sequenced a majority of the phaseolotoxin gene cluster, except for locus A and a small portion of locus B. In at least one locus (*phtF*) we have found a DNA-binding motif. The protein(s) produced by the organism at 30°C could be involved in binding to this motif leading to the repression of the gene.

b) We have purified one of the proteins (37 kD) produced at 30° C by *P.s.*pv. *phaseolicola*, and have cloned the gene that encodes this protein. Amino acid alignments have shown that the 37 kD protein has 54% identity with a protein CbpA encoded by the *cbpA* gene of *E.coli*. CbpA of *E.coli* was initially isolated and shown to bind to curved DNA (Yamada et al., 1990). CbpA of *E. coli* has high homology to DnaJ protein of *E.coli*. Both proteins are considered to be heat-shock proteins and have been shown to be involved in DNA synthesis and other functions of the cell. DnaJ is a chaparone of DnaK. It is very interesting that *P.s.*pv.*phaseolicola* also produces a Cbp-like protein at higher than normal growth temperature, and may be considered a heat-shock protein which has an additional function as a repressor of *argK* and some of the phaseolotoxin biosynthetic genes.

References

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Kamadar, H.V., Rowley, K.B., Clements, D. and Patil, S.S. 1991. *Pseudomonas syringae* pv. phaseolicola genomic clones harboring heterologous DNA sequences suppress the same phaseolotoxin-deficient mutants. J. Bacteriol. 173:1073-1079.

Rowley, K. B., Clements, D. E., Mandel, M., Humpreys, T. and Patil, S.S. 1993. Multiple copies of a DNA sequence from Pseudomonas syringae pathovar phaseolicola abolish thermoregulation of phaseolotin production. Molecular Microbiology 8:625-635.

Rowley, K.B., Xu, R. and Patil, S. S. 2000. Molecular analysis of thermoregulation of phaseolotoxin-resistant ornithine carbamoyltransferase (*argK*) from *Pseudomonas syringae pv. phaseolicola*. Molecular Plant-Microbe Interactions. 13(10):1071-1080.

Xu, R., Rowley, K. B., Mocz, G. and Patil, S. S. 1999. Purification of a putative repressor involved in the thermoregulation of phaseolotoxin-resistant ornithine carbamoyltransferase (ROCT) of *Pseudomonas syringae pv. phaseolicola.* Phytopathology. 89:S86 (abstr).

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Zhang, Y. X. and Patil, S.S. 1997. The *phtE* locus in the phaseolotoxin gene cluster has ORFs with homologies to genes encoding amino acid transferases, the AraC family of transcriptional factors, and fatty acid desaturases. Mol. Plant-Microbe Interact. 10: 947-969.

Personnel:

Two graduate students joined the lab in September 1996. Chenyang Xu (Beijing University) changed fields and went to another University on the mainland in December 1996. Lee Wang (Shanghai University) left to join her husband at another University on the mainland in May 1997. This set the project back considerably. ORA granted a no cost extension up to 04/30/2000. Two new students, L. Ratnapala (Ph.D.) and R. Xu (M.S.) joined the lab. Ratnapala worked on objective 3 above and Xu devoted his time to objective 1. Xu completed his M.S. degree in June 1999 and rejoined the lab as Research Associate in July 1999. Ratnapala has not completed the requirments for a Ph.D. degree.

The following is the summary of the periods during which they were supported by the ARO grant.

Graduate Research Assistants:

1) Chenyang Xu: September 1996-December 1996.
2) Lee Wang: September 1996-May 1997.
3) L. Ratnapala: February 1997-October 1998 (50% support from ARO grant + 50% from the University of Hawaii, PBRC).
4) Ronghui Xu: February 1997-June 1999 (Graduated with a M.S. degree in Cell and Molecular Biology Graduate program).

Casual Hire (Full time):

Ronghui Xu: 7/21/99 through 9/20/99, Research Associate (Full time technician): 9/21/99 through 7/31/00

Two undergraduate students, Carol Inatsuka and Charmaine San Jose, worked as laboratory assistants during the summer months (July-August 2000) to help with the screening of the library and other tasks.

List of Publications, Reports and Abstracts

Publication

Rowley, K.B., Xu, R. and Patil, S. S. 2000. Molecular analysis of thermoregulation of phaseolotoxin-resistant ornithine carbamoyltransferase (*argK*) from *Pseudomonas syringae pv. phaseolicola*. Molecular Plant-Microbe Interactions. 13(10):1071-1080.

Abstracts

Xu, R., Rowley, K. B., Mocz, G. and Patil, S. S. 1999. Purification of a putative repressor involved in the thermoregulation of phaseolotoxin-resistant ornithine carbamoyltransferase (ROCT) of *Pseudomonas syringae pv. phaseolicola.* Phytopathology. 89:S86.

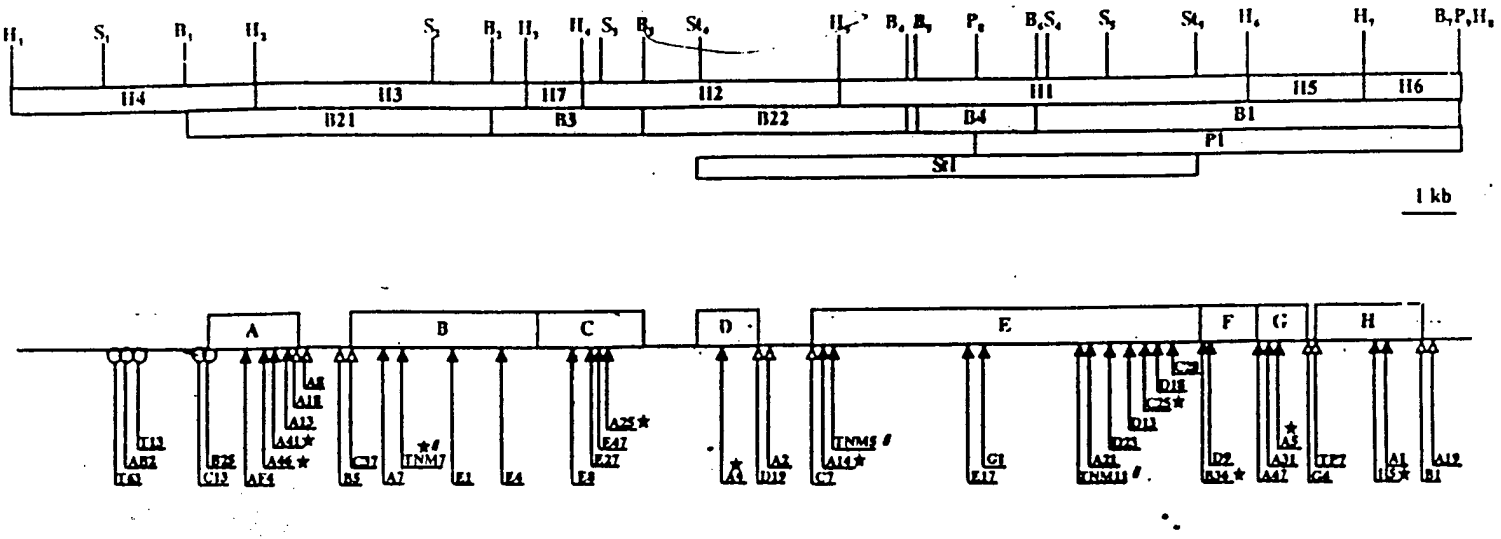
Ratrapala, L. and Patil, S. S. 1998. DNA sequence analysis of genes in the phaseolotoxin gene cluster with respect to homologies to known genes and to determine whether thermoregulation regions exsist. Biomolecular signalling, Energy transfer and Transduction processes; Physiology and Performance. A workshop-symposium sponsored by the U.S. Army Research Office, Biological Sciences Division, Research Triangle Park, NC 27709, May 4-6, 1998, Cashiers, NC

Other

A minimum of three mannscripts will be submitted to peer reviewed journals in the coming months, based on the results reported in this final report. Previously(1997, 1998), I have submitted interim reports. No interim report for 1999 was submitted because of the no-cost extension of the grant to 7/30/2000. This terminal report covers the entire period of the grant.

APPENDIX

Fig 1A Physical map of Phaseolotoxin Gene Cluster (Zhang *et al.*, 1997)



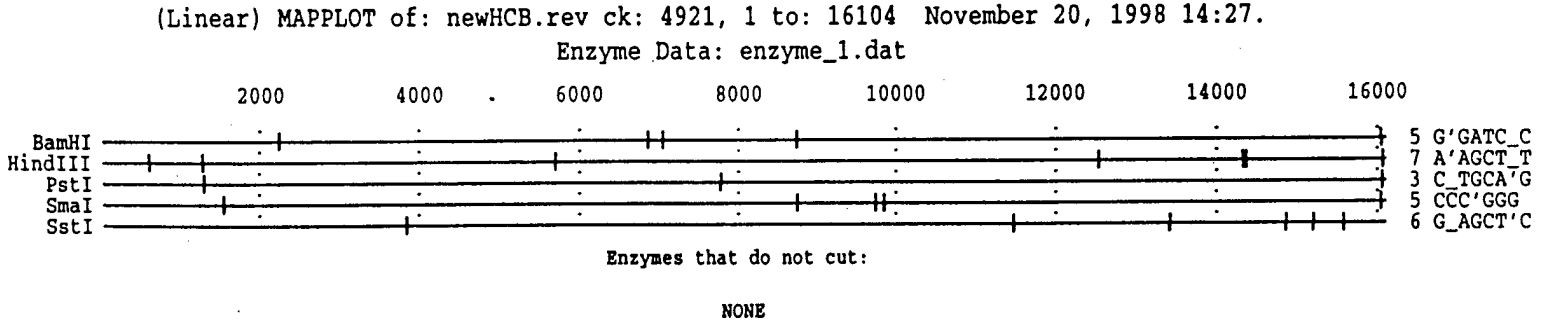


Fig 1B Restriction site marker of DNA sequence of current study

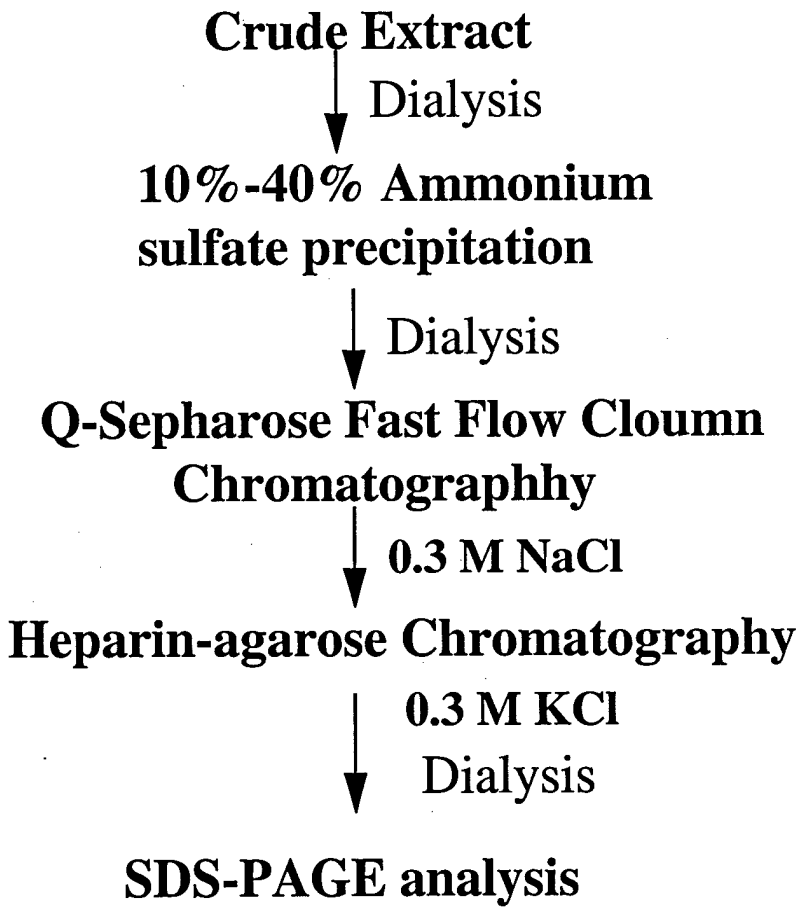


Fig. 2 Purification Scheme for the isolation of TP

SDS-PAGE analysis of 0.3 M KCl Extract from Heparin-agarose Column

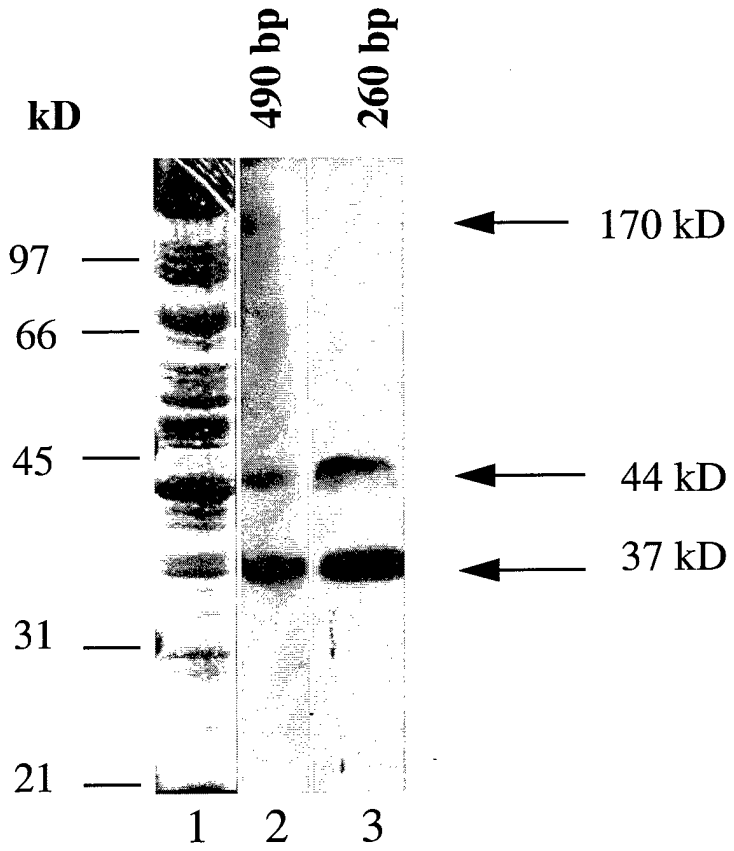


Fig. 3 Lane 1: Protein markers(Bands stained with Coomassie R-250).

Lane 2: 490 bp fragment from TRR as probe(Southwestern blot).

Lane 3: 260 bp subfragment of the 490 bp fragment as probe (Southwestern blot).

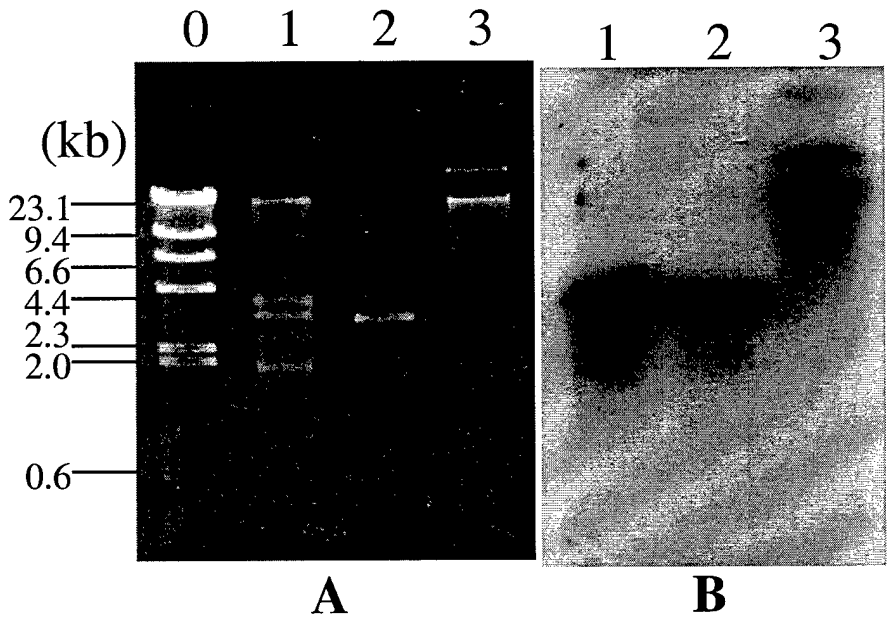


Fig. 4 Gel electrophoresis and Southern blot analysis of the pLAFR3 Library clone.
A. Gel electrophoresis.
B. Southern blot of A.

Lanes: 0) DNA ladder.
1) pLAFR3 clone digested with *Pst*I.
2) Recovered fragment from *Pst*I digest from lane 1.
3) pLAFR3 library clone without digestion.

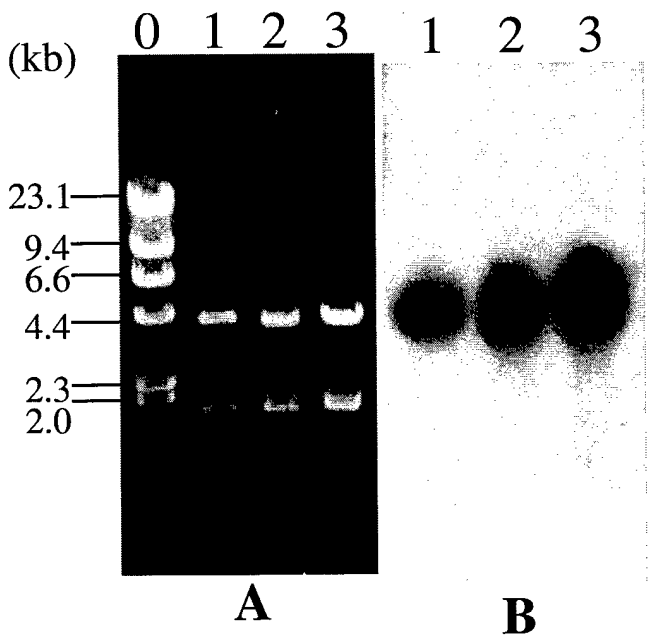


Fig. 5 Identification of plasmid pRX containing the 3 kb insert.
A. Restriction analysis of three plasmid clones(isolated from three individual colonies digested with *Sca*I-*Hind*III) following agarose gel electrophoresis.
B. Southern blot of A.

Lanes:
0) DNA ladder
1) pRX 1 (pT7/T3α–19 clone)
2) pRX 2 (pUC19 clone)
3) pRX 3 (pUC19 clone)

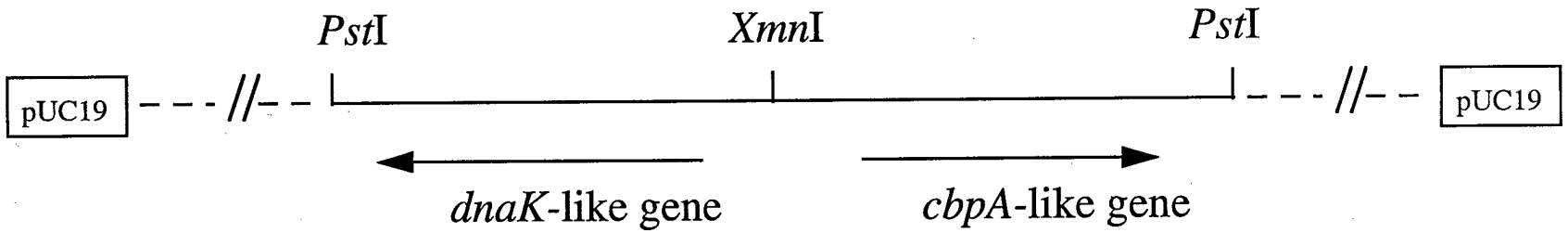


Fig. 6 Location of *cbpA*-like and *dnaK*-like genes on plasmid pRX 3.

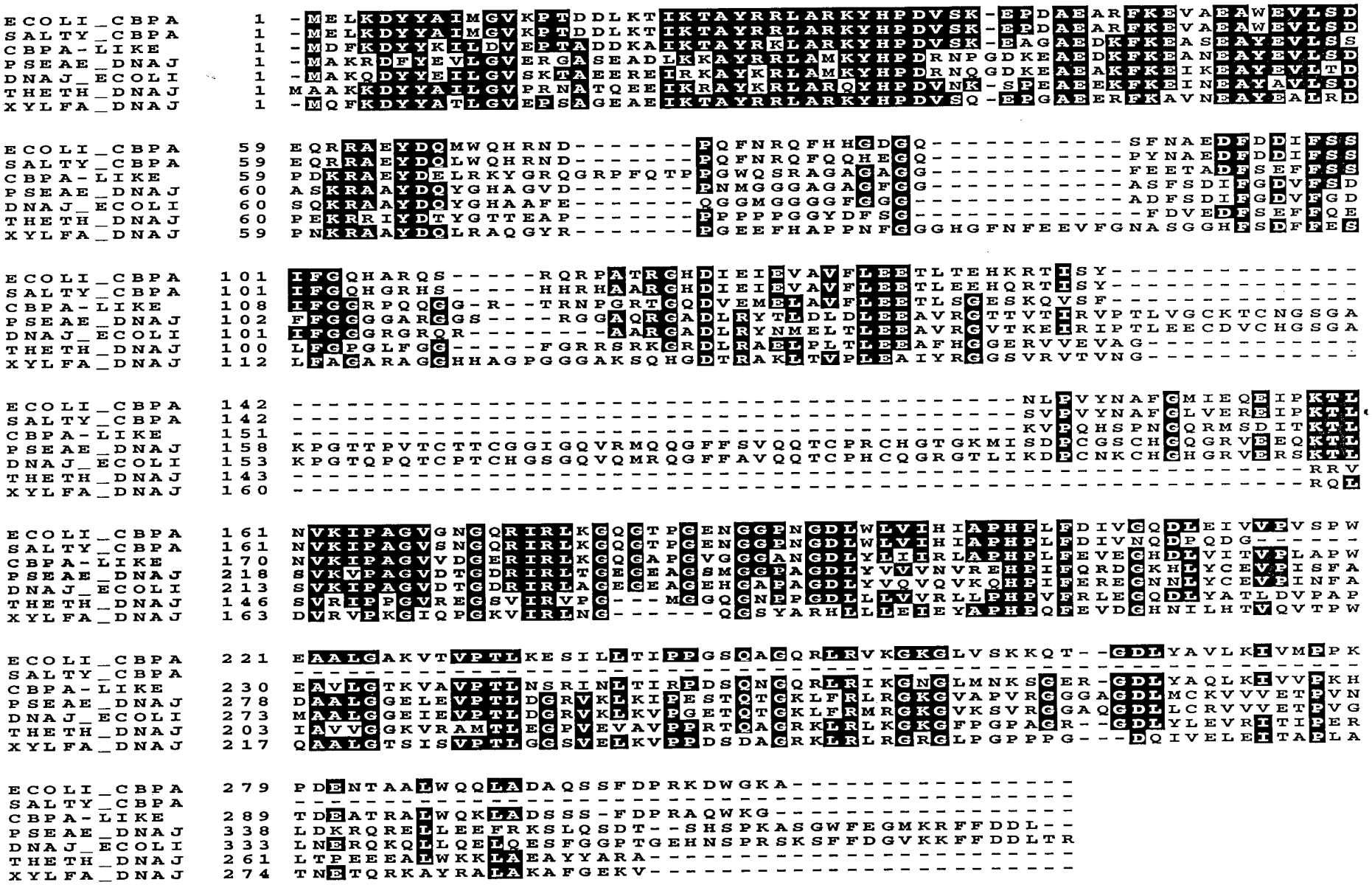


Fig. 7 Amino acid sequence alignments. *P.s. pv. phaseolicola* CbpA-like sequence was compared with *E. coli* CbpA, *Salmonella typhimurium* CbpA, *Pseudomonas aeruginosa* DnaJ, *E. coli* DnaJ, *Thermus thermophilus* DnaJ and *Xylella fastidiosa* DnaJ.

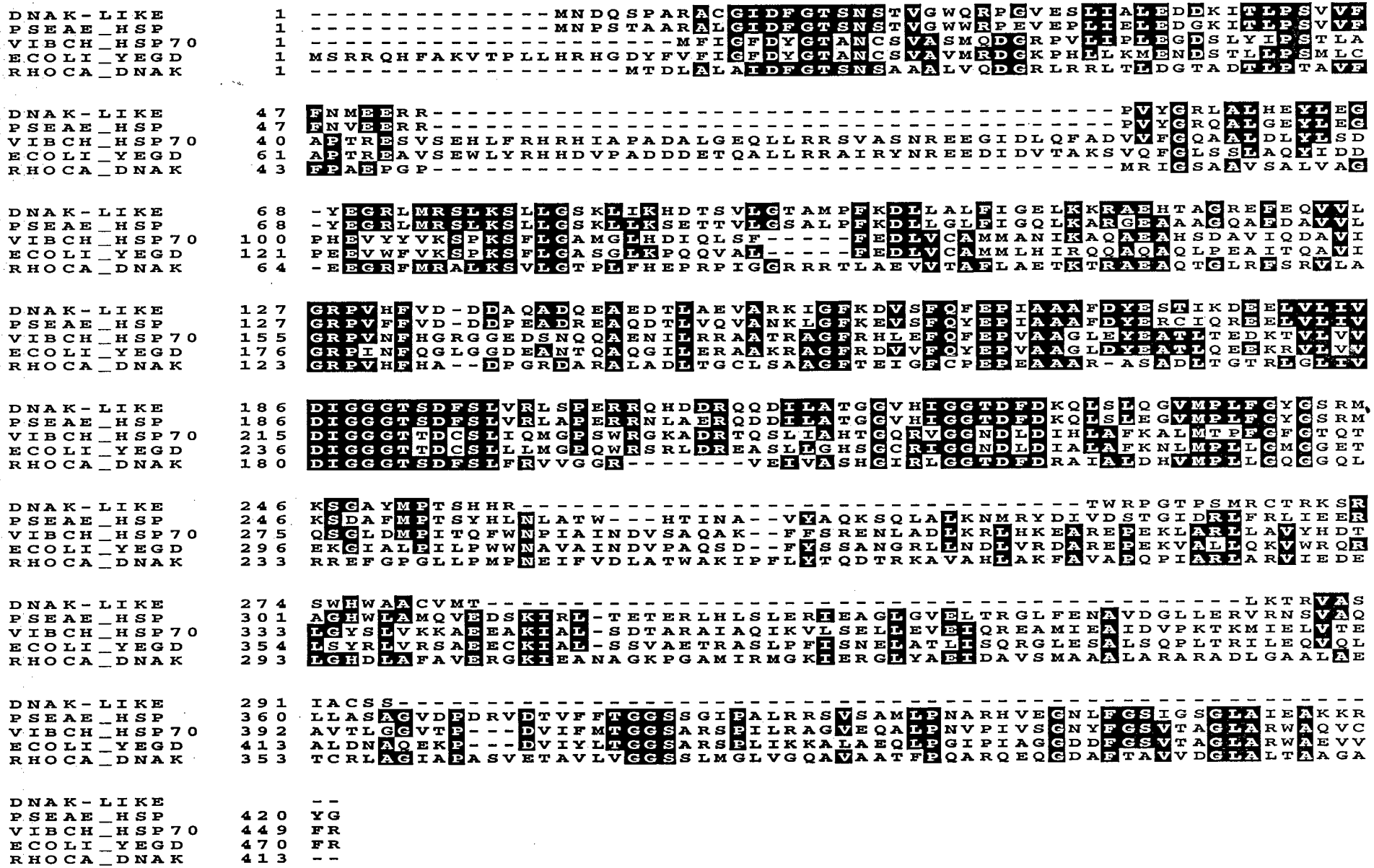


Fig. 8 Amino acid sequence alignments. ***P.s. pv. phaseolicola*** **DnaK-like sequence was compared with** ***Pseudomonas aeruginosa*** **heat-shock protein,** ***Vibrio cholerae*** **heat shock protein 70 family protein,** ***E. coli*** **YegD and** ***Rhodobacter capsulatus*** **DnaK.**